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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	§	
Mar Tormo	§	
Ana M. Tari	§	
Gabriel Lopez-Berestein	§	
	§	Group Art Unit: 1805
Serial No. 08/726,211	§	
	§	Examiner: R. Schwartzman
Filed: October 4, 1996	§	
	§	Atty. Dkt.: UTXC:504/COD
For: INHIBITION OF BCL-2 PROTEIN	§	
EXPRESSION BY LIPOSOMAL	§	
ANTISENSE	§	
OLIGODEOXYNUCLEOTIDES	§	

**DECLARATION OF GABRIEL LOPEZ-BERESTEIN AND ANA M. TARI
UNDER 37 C.F.R. §1.131**

WE, DR. GABRIEL LOPEZ-BERESTEIN AND DR. ANA M. TARI DECLARE AS
FOLLOWS:

1. We, along with Dr. Mar Tormo, are co-inventors of the subject matter of the captioned patent application USSN 08/726,211.
2. It is our understanding that the Examiner in charge of the captioned application has rejected claims 1, 2, 5 and 6 as being anticipated by the abstract of Tormo *et al.* abstract #1190, *Proceedings of the American Association for Cancer Research*, vol. 37 pg 173, March 1996, and has rejected claims 1 and 2 as anticipated by the abstract of Almazan *et al.* abstract #2407, *Proceedings of the American Association for Cancer Research*, vol. 37 pg 353, March 1996.

3. Our invention was made and tested in this country prior to March 1996 and thus prior to the publication of the cited abstracts.

4. The fact that our invention was made and tested in this country prior to March of 1996 is evidenced by studies set forth in the attached notebook pages 280-288 of the laboratory notebook of Mar Tormo. Among other things, this notebook sets forth the following studies which exemplify the practice of our invention:

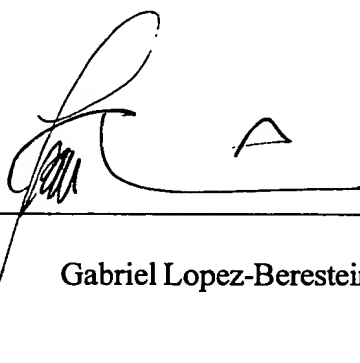
- a) measurement of expression of Bcl-2 in Johnson, Daudi, Raji and Jurkat cells (p. 280);
- b) a test of growth inhibition of the cell lines with liposomal AS (antisense), C (control) and B₂ oligonucleotides (p. 280-281);
- c) graphs depicting apoptotic index of cells and cell density after treatment with control and antisense oligonucleotides (p. 282);
- d) a repeat of the study of measurement of expression of Bcl-2 in Johnson, Daudi, Raji and Jurkat cells (p. 282-283);
- e) growth inhibition studies of Johnson, Daudi, Raji and Jurkat cells in the presence of antisense and control liposomal oligonucleotides.

Each of items a) through e) as represented in the attached notebook were carried out in this country prior to March 1, 1996.

5. All statements made in this Declaration of my own knowledge are true and all statements made in this Declaration on information and belief are believed to be true, and these statements are made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both under 18 U.S.C. §1001 and may jeopardize the validity of this application or any patent issuing thereon.

1-8-98

Date



Gabriel Lopez-Berestein

1-7-98

Date

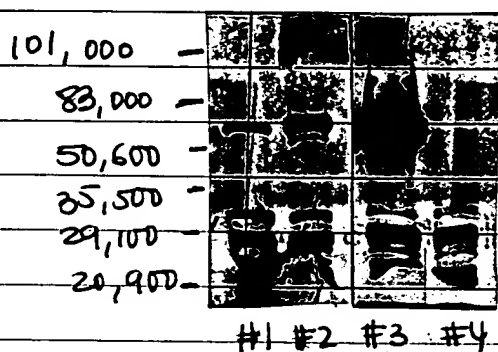


Ana M. Tari

Sample	diluted $\frac{1}{2x}$ with 2x SB	for 5 μ g	SB by 10 20 μ l
Daudi	3.042	8.2 μ l	11.8 μ l
Johnson	3.104	8.05 μ l	11.95 μ l
Jurkat	4.712	5.3 μ l	14.7 μ l
Raji	4.651	6.4 μ l	14.6 μ l

\Rightarrow Blotting:

p53 : 1/1000 (2nd anti-mouse 1/4000)
Bcl-2 : 1/1000 (2nd anti-hausta 1/4000)



#1 Johnson
#2 Daudi
#3 Raji
#4 Jurkat

Wed

Monday 18 Growth inhibition exp # 26

\rightarrow Test all cell lines with AS, C and B₂ lipids in liposome

Cell density do = 10,000/well in 100 μ l

Doses: 2, 3, 4, 5, 6, 7 and 8 μ M

\rightarrow Sonicate liposomes 12' and measure

(AS) (Microp distribution)

	Peak 1	Peak 2	RT Error 44.3
WL	36.3 (56.1%)	241.8 (44.1%)	Residual 0.00
INT	36.8 (41.6%)	238.4 (58.4%)	
NVM	34.7 (99.7%)	231.5 (0.3%)	

(C)

			RT Error: 39.98
WL	35.8 (55.8%)	172.2 (44.2%)	Residual: 0.00
INT	35.8 (34.6%)	170.7 (65.4%)	
NVM	33.8 (99.8%)	104.8 (0.7%)	

SB for (13)

20 μ l

B213

11.8 μ l11.95 μ l14.7 μ l14.6 μ l

VOL: 29.5 (56.9%) 110.2 (43.1%) Fit Error 23.14
 INT: 29.4 (20.6%) 108.7 (69.4%) Residual 50.29
 NM: 28.0 (98.5%) *

→ Add dyes to the cells. Incubate 5 day
(day 5 → Saturday 23)

mouse 1/4000
hamster 1/4000

(2) Freeze Jurkat and Daudi cells
 (see P#2)
 Jurkat → 7 tubes (with 4×10^6)
 Daudi → 9 " (with 3×10^6)

at Wednesday 20 (1) Prepare new vials: 40 μ moles
of vials with 400 μ moles of lipids

$$A : \frac{40 \times 10^3 \text{ pmoles}}{1270 \text{ pmoles } \mu\text{l}} \rightarrow 31.5 \mu\text{l}$$

$$C : \frac{40 \times 10^3 \text{ pmoles}}{662 \text{ pmoles } \mu\text{l}} \rightarrow 60 \mu\text{l}$$

$$\text{Lipids: } \frac{0.4 \mu\text{moles} \times 786.12 \mu\text{g } \mu\text{mole}^{-1}}{30 \mu\text{g } \mu\text{l}^{-1}} \rightarrow 10.5 \mu\text{l}$$

AS, C and

1 well in 100 μ l8 μ M

and measure:

nm

Fit Error 44.3

Residual 0.00

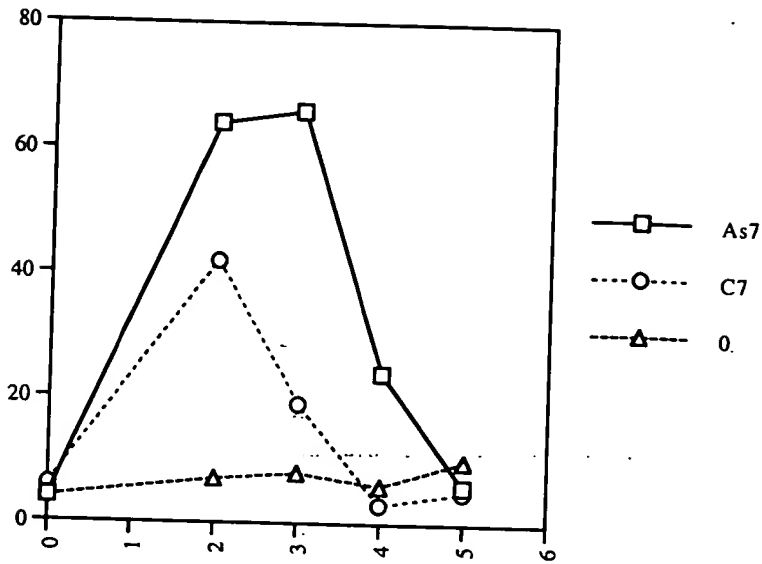
	AS	C	Empty
lipids	10.5	10.5	10.5
vial	31.5	60	0
t-butanol	2ml	2ml	1.5ml

(*) from pag 276

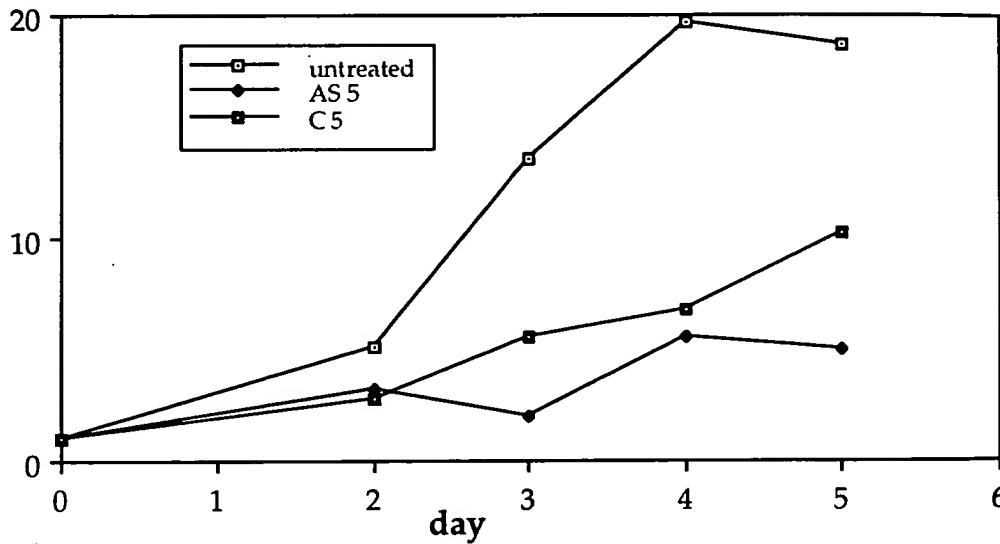
Fit Error: 39.98

Residual: 0.00

Apoptotic Index, %



($\times 10^5$)



204,000

121,000

82,000

50,200

34,200

28,100

19,400

7,350

12/24/01

② I will go to run again the gel with extract of the four cells lines untreated for to test the bcl-2 expression because in the previous blot there are many unspecific bands and a bad transference.

Sample	diluted 1/2 with 2x SB	ul for 25 μ g	ul for 50 μ g
Johnson	3.104	8.1 μ l	16.2 μ l
Daudi	3.042	8.2 μ l	16.4 μ l
Jurkat	4.712	5.3 μ l	10.6 μ l
Raj	4.651	5.4 μ l	10.8 μ l

204,000

121,500

82,000

50,200

34,200

28,100

19,400

7,350

1. Johnson 5
2. Daudi 6
3. Jurkat 7
4. Raj 8

w

25 μ g

w

50 μ g

1 2 3 4 5 6 7 8

12/24/95 Growth inhibition exp # 26. day 5
 Blaumar blue Assay
 Results:

① Johnson

	2	3	4	5	6	7	8	ϕ	Blank		
AS	0.762	0.725	0.108	-0.164	-0.241	-0.242	-0.249	0.792	-0.244		
	0.761	0.624	-0.035	-0.213	-0.250	-0.261	-0.250	0.742	-0.247		
C	0.726	0.721	0.705	0.530	0.196	-0.104	-0.229	0.736			
	0.725	0.788	0.729	0.625	0.327	-0.175	-0.218	0.764			
B ₂	0.788	0.758	0.736	0.794	0.634	0.329	0.124	0.721			
	0.776	0.784	0.732	0.739	0.453	-0.053	-0.090	0.764			

AS

C

B₂ (% viability)

2

100%

96%

104%

3

89%

100%

102%

4

5%

95%

97%

5

0%

76%

101%

6

0%

35%

72%

7

0%

0%

18%

8

0%

0%

2%

with

utrea

iru

areap

bad

1 for

0 μ g.2 μ g.4 μ g.6 μ g.8 μ g

iability

AS C B₂ (98% variability)

2	98%	100%	104%
3	96%	98%	97%
4	85%	92%	99%
5	33%	77%	95%
6	0%	35%	91%
7	0%	0%	87%
8	0%	0%	72%

(4) Daudi

	2	3	4	5	6	7	8	Ø	Block		
AS	0.460	0.351	-0.123	-0.202	-0.219	-0.222	-0.219	-0.481	-0.227		
	0.533	0.352	0.225	-0.215	-0.221	-0.217	-0.220	0.498	-0.229		
C	0.489	0.463	0.337	0.076	-0.203	-0.213	-0.222	0.516			
	0.503	0.451	0.281	0.170	-0.148	-0.220	-0.215	0.510			
B ₂	0.514	0.463	0.419	0.387	0.238	-0.099	-0.103	0.458			
	0.498	0.461	0.470	0.388	0.289	-0.021	-0.144	0.520			

AS C B₂ (90% variability)

2	97%	87%	89%
3	62%	80%	81%
4	39%	54%	78%
5	0%	21%	68%
6	0%	0%	46%
7	0%	0%	0%
8	0%	0%	0%

10/10/11 WED Sep 14 6

- ① To test if there are bel-2 inhibitors with AS. Jones: 2, 3, 4, 5, 6 and 7 of AS and C oligo
- ② Prepare the oligo like always and sonicate for 12' (4' - 4' - 4')
- ③ After measurement of the liposomes site:

F-ANN UPTAKE

Menu File: C370.TBL 1/3/96

AS

Run time		Data Ch		Intensity		Sensitivity	
0 Hours	7 Mins 48 Secs	131.7K	1.5kHz	132	255		
Gaussian Analysis		(Vesicle Distribution Analysis)					
Mean Dia	Std Dev	Chi Sq	Peak 1	Peak 2	Peak 3		
VOL: 152.3 nm	112.7 nm	297.42	12.5 %	35.5	192.0	Fit Error	
	74.0 %		5.4 %	54.1 %	40.5 %	5.813	
INT: 133.2 nm	98.5 nm	297.42	12.7 %	36.1	176.8	Residual	
	74.0 %		1.3 %	35.7 %	63.1 %	51.909	
NUM: 24.8 nm	18.3 nm	297.42	29.1	169.6			
	74.0 %		99.4 %	0.6 %			

F-ANN UPTAKE

Menu File: C370.TBL 1/3/96

C

Run time		Data Ch		Intensity		Sensitivity	
0 Hours	9 Mins 24 Secs	37.9K	.4kHz	101	255		
Gaussian Analysis		(Vesicle Distribution Analysis)					
Mean Dia	Std Dev	Chi Sq	Peak 1	Peak 2	Peak 3		
VOL: 76.0 nm	51.3 nm	4.44	26.0 %	80.7		Fit Error	
	67.5 %		30.1 %	69.9 %		15.788	
INT: 87.2 nm	58.8 nm	4.44	27.9	81.7		Residual	
	67.5 %		14.6 %	85.4 %		47.565	
NUM: 18.3 nm	12.3 nm	4.44	21.4	63.4			
	67.5 %		92.1 %	7.9 %			

- ④ Add to the cells (Johnson's cells - plated in a 24 well plate at the 100,000 cells/well, 1 ml in each well)

- ⑤ Incubate for 3 days.

② Check again the belz of my four cell²⁸⁷ lines (in previous blott it was very dirty). Prepare new supernatant of Raji and Jurkat cells and measure the amount of protein again.

Curve Fit: Linear
Equation: $y = A + B \cdot x$
A = 0.00769 B = 0.287
Corr. Coeff: 1.00
Std Units: ug/ul

PLATE BLANK	Well	OD	Mean	Std Dev	CV
BL	A12	0.053	0.053	*****	*****

STANDARDS	Value	Well	OD	Mean	Std Dev	CV
STD01	0.000 ug/ul	A1	0.017	0.012	0.005	38.19
		B1	0.008			
		C1	0.011			
STD03	0.500 ug/ul	A3	0.186	0.144	0.050	34.57
		B3	0.156			
		C3	0.089			
STD04	0.750 ug/ul	A4	0.398	0.224	0.157	70.21
		B4	0.182			
		C4	0.092			
STD06	1.500 ug/ul	A6	0.588	0.440	0.131	29.70
		B6	0.389			
		C6	0.342			

UNKNOWN	Mean	Std Dev	CV	Well	Value	OD	Dil. Factor
DAUDI	1.940	0.264	13.61	E3	1.754	0.058	10.00
				F3	1.824	0.060	
				G3	2.243	0.072	
JOHNS	1.754	0.160	9.108	E1	1.929	0.063	10.00
				F1	1.615	0.054	
				G1	1.720	0.057	
JURK	1.603	0.053	3.322	E2	1.615	0.054	10.00
				F2	1.650	0.055	
				G2	1.545	0.052	
RAJI	2.836	0.126	4.434	E4	2.731	0.086	10.00
				F4	2.801	0.088	
				G4	2.975	0.093	

Line	Sample	Value	Volume
1	Johns	0.877	40 µl
2	Dauidi	0.97	36 µl
3	Jurkat	0.802	44 µl
4	Raji	1.420	25 µl

288

Blot 1^o (Bd-2) 1/1000

(5)

PLATE BL
BLSTANDARD
STD01

STD02

STD03

STD04

STD05

STD07

UNKNOWN
0

AS2

AS3

AS4

AS5

AS6

C2

C3

C4

C5

C6

1/8/96

WB exp #6

1) Count the cells manually:

$$\phi = 1.065 \times 10^6 \text{ (99\% viab.)}$$

$$2AS = 0.845 \times 10^6 \text{ (99\%)}$$

$$3AS = 0.580 \times 10^6 \text{ (91\%)}$$

$$4AS = 0.325 \times 10^6 \text{ (92\%)}$$

$$5AS = 0.555 \times 10^6 \text{ (89\%)}$$

$$6AS = 0.170 \times 10^6 \text{ (55\%)}$$

$$2C = 1.205 \times 10^6 \text{ (96\%)}$$

$$3C = 0.975 \times 10^6 \text{ (98\%)}$$

$$4C = 0.645 \times 10^6 \text{ (97\%)}$$

$$5C = 0.385 \times 10^6 \text{ (94\%)}$$

$$6C = 0.190 \times 10^6 \text{ (76\%)}$$

2) Lysed the cells (protocol)

3) Normalization of amount of protein

2.5 μ g the gel 3% 3

4) Transfer o/n 33 wt